# THE INFLUENCE OF BLOOD MEAL ON SUSCEPTIBILITY TO PYRETHROIDS IN *ANOPHELES GAMBIAE* FROM BUNGOMA, WESTERN KENYA

**BY**

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# DECLARATION

I declare that this thesis is my original work and has not been presented to any other University or Institution for a degree or any other award.

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May God bless you all!!

## DEDICATION

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## ABSTRACT

Malaria continues to be an important disease in the tropics posing a major obstacle to sustainable development. Malaria control is dependent on pyrethroid insecticides yet resistance to these insecticides in the malaria vector *Anopheles gambiae* is receiving increasing attention because it threatens the sustainability of malaria vector control programs targeted at indoor resting mosquitoes in Western Kenya. Common mechanism of resistance reported to these insecticides is target site insensitivity and through degradation of the insecticides by metabolic enzymes. When a female mosquito takes a blood meal, altered gene expression occurs in order to accommodate and utilize the nutrients; hence, it is hypothesized that the enzymes responsible for the detoxification of xenobiotics in the blood meal may influence the subsequent level of susceptibility to insecticides following exposure. The aim of this study was to determine the influence of blood meal status on deltamethrin tolerance in wild collected (Bungoma) *Anopheles gambiae* alongside Kisumu susceptible strain of *Anopheles gambiae* as a reference strain. WHO-tube susceptibility test was done to determine the difference in susceptibility to deltamethrin in *Anopheles gambiae* with different gonotrophic status at different age groups (2-4 and 14-16 days old) from the two populations. Metabolic assays were done to measure change in levels of detoxification enzymes (Oxidase, non-specific esterases and Glutathione- S- transferases), in response to the presence of a blood meal. The Mortality rates were calculated as a percentage of individual mosquitoes that died within 24 hours of exposure and levels of resistance were classified according to WHO guidelines. All means were compared using either a 2-sample t-test or a 1-way Analysis of Variance (ANOVA) with a Tukey comparison-of-means as a post-hoc test. All confidence intervals were set at 95%.Statistical analysis was done using SPSS version 21.0. Bioassay results showed younger (2-4 days) unfed wild mosquitoes were more resistant to pyrethroids than older (14-16) unfed ones (Mortality rates 83% vs 98%) which was significantly different ($p < 0.047$). Results showed reduced mortality in younger wild collected mosquitoes with various gonotrophic status (mortality ranged from 36-83%). Older females from the same population with varying gonotrophic status showed increased mortality to the same insecticide (85-98%). Kisumu susceptible population showed 100% susceptibility independent of their gonotrophic status. Biochemical estimations on the wild population revealed significantly ($P<0.05$) higher levels of oxidase, non-specific esterase and glutathione-S-transferases activity in the blood fed and half gravid survivors *of An. gambiae* as compared to unfed survived younger individuals. For older females from the same population, blood fed and half gravid survivors showed significantly higher oxidase and glutathione-S-transferases activity as compare to unfed and gravid survivors. For the Kisumu susceptible strain, oxidases , non-specific esterases and GSTs levels were higher in both younger and older fed groups compared to the unfed groups ($P<0.05$). Thou there was no significant difference in elevation of all the three enzyme measured between the means of unexposed live groups and the exposed knocked down groups for both age groups ($P>0.05$). The results showed that blood feeding in vector mosquitoes plays an important role in the toxicity of deltamethrin. It is believed that this change in susceptibility may be due to increasing systemic expression of detoxification enzymes as metabolic activity increases during the process of blood digestion. This enzymes may also confer increased tolerance of blood fed mosquitoes to insecticides. These may have implications for the sustained efficacy of the indoor residual spraying and insecticide treated nets based control programs that target indoor resting female mosquitoes of various gonotrophic status.

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## LIST OF ABBREVIATIONS AND ACRONYMS

**AChE1**- Acetylcholinesterase

**CDC** – Centers for Disease Control and Prevention

**CYP6z1** – Cytochrome P450, gene family 6, sub family z, gene 1

**CYP6m2** – Cytochrome P450, gene family 6, sub family m, gene 2

**DDT** - Dichloro-diphenyl-trichloroethane

**GST** – Glutathione-S-Transferase

**GABA**- Gamma-Aminobutyric Acid

**IRS** – Indoor Residual Spraying

**ITNs**- Insecticide Treated Nets

**Kdr**- Knock-down resistance

**KEMRI**- Kenya Medical Research Institute

**LLINs**- Long Lasting Insecticide treated Nets

**MFO**- Mixed Function Oxidases

**NSE**- Non-Specific Esterases

**WHO** – World Health Organization

## DEFINITIONS OF TERMS

**I. Insecticide resistance (WHO):** Development of an ability or strain of some organisms to tolerate doses of a toxicant that would prove lethal to a majority of individuals in a normal population of the same species.

**II. Insecticide target site resistance:** changes in the sensitivity of insecticide target due to non-silent point mutations.

**III. Metabolic resistance:** over-expression of enzymes capable of detoxifying or sequestering insecticides and/or amino acid substitutions within these enzymes which alter the affinity of the enzyme for the Insecticide.

**IV. Phenotypic resistance:** expression of a resistance gene on an insect life history trait (e.g. survival rate, knock down, etc.).

**V. Gonotrophic cycle :** Mosquito Life cycle of alternate blood feeding and laying eggs. " Reproductive feeding"

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# CHAPTER ONE: INTRODUCTION

## 1.1 Background information

*Anopheles gambiae s.l* is the major vector of malaria transmission in sub-Saharan Africa where the disease remains the most important parasitic disease causing significant morbidity and mortality despite concerted efforts to control it (WHO, 2010). Globally it is estimated in the year 2013 that 3.3 billion people are at risk of being infected with malaria and developing disease, and 1.2 billion are at high risk (>1 in 1000 chance of getting malaria in a year) (WHO, 2014). According to the latest estimates , 198 million cases of malaria occurred globally in 2013 and the disease led to 584 000 deaths, representing a decrease in malaria case incidence and mortality rates of 30% and 47% since 2000, respectively (WHO, 2014). The overwhelming global burden of malaria is largely concentrated in the tropics and particularly in sub-Saharan Africa owing to environmental risk factors that favor transmission of *Plasmodium* parasites (WHO, 2008). The burden is heaviest in this region where an estimated 90% of all malaria deaths occur, and in children aged under 5 years, who account for 78% of all deaths (WHO, 2014).

In Kenya, approximately 25 million people are exposed to stable malaria transmission every year, including 3.5 million children below the age of 5 years (KMIS, 2010). Case fatality is very high among children with an estimated death toll of 26,000 per year (WHO, 2010). In the tropics, malaria transmission is mainly dominated and driven by four important vector species that are ecologically adapted to reproduce and survive in the area. The predominant malaria vectors include *Anopheles gambiae* complex (*Anopheles gambiae s.s, Anopheles arabiensis and anopheles merus*) and *Anopheles funestus* complex. *An. gambiae s.l* being the main malaria vector in region (WHO, 2004). Among the drivers of malaria in this region are the climate

change and variability. For instance temperature and rainfall, affect the development of mosquitoes and malaria parasites (WHO, 2004). Increasing temperature accelerates the rate of mosquito larval development, the frequency of blood feeding by adult females on humans, and reduces the time it takes the malaria parasites to mature in female mosquitoes (Githeko and Ndegwa, 2001).

Although malaria is both a curable and preventable disease with many interventions available, its control in sub-Saharan Africa is constrained by poverty and insufficient health infrastructures(Sachs and Melaney, 2002). Rural and poor people suffer the burden of the disease due to limited treatment; as a result infection rates are highest in the rural during rainy season because this is a time of high agricultural activities that provide more breeding grounds for the *Anopheles* mosquitoes that transmit malaria (Sachs and Melaney, 2002).

The major obstacles to malaria control are the absence of a protective vaccine, the spread of parasite resistance to anti-malarial drugs and the mosquito resistance to insecticides (Hemingway and Ranson, 2000; Trape, 2001). Vector control and surveillance are important components of malaria reduction and control which aims at interrupting the malaria parasite transmission cycle (Djènontin *et al*., 2009). The primary measures that have been employed for malaria vector control are the application of Indoor residual spraying (IRS) and the use of pyrethroid-treated bed nets (ITN). Currently, synthetic pyrethroids i.e. Permethrin, Deltamethrin and Lambdacyhalothrin are the preferred group of insecticides recommended for use in ITNS and IRS owing to their strong insecticidal activity at low concentrations and their low mammalian toxicity (WHO, 2004). Kenya has made significant progress in scaling up malaria control efforts, with targeted subsidies of ITNs beginning in 2003, a mass distribution of ITNs targeting children aged <5 years in 200 and a universal coverage campaign in 2011 which provided one long

lasting insecticide treated net (LLIN) for every two people (Okiro *et al*., 2010; Zhou *et al*., 2011). In addition from 2006, through funding from the Global Fund for HIV and AIDS, Tuberculosis and malaria, indoor residual spraying (IRS) was started in selected districts in western Kenya which are prone to epidemics (DOMC, 2011). Insecticide treated nets (ITNs) and indoor residual insecticide spraying (IRS) are the cornerstones of malaria vector control which target mostly the indoor resting mosquitoes(WHO, 2008).

Although these control measures appear successful in reducing malaria prevalence, the challenges facing the use of insecticides for malaria vector control is the development of resistance which has been reported in West and Central African and East African countries including Western parts of Kenya (Matowo *et al*., 2010; Mathias *et al*., 2011; Ochomo *et al*., 2014). Due to this challenge, several studies have been carried out to understand the evolution and mechanism of resistances which impacts negatively on malaria control in Western Kenya (Martinez-Torres *et al*., 1998; Vulule *et al*., 1999; Kamau *et al*., 2008; Ochomo *et al*., 2014; Wanjala *et al*., 2015).

Insecticide resistance is predominantly based on overproduction of detoxifying enzymes that sequester and/or degrade the insecticide before it reaches the nervous system (metabolic resistance) and mutations in the insecticide neural targets that render them less sensitive to the insecticide's active ingredient (target site resistance) (Hemingway *et al*., 2004). Different studies have linked Pyrethroid resistance in *Culex pipiens pipiens*, *An. gambiae* and *An. funestus* to increased activity of cytochrome P450s, members of the monooxygenase class of detoxification enzymes (Brooke *et al*., 2001; McAbee *et al*., 2003; Nikou *et al*., 2003; Amenya *et al*., 2008). Further, the P450 monooxygenases have been implicated in the detoxification of xenobiotics

including drugs, pesticides and plant toxins as well as endogenous metabolic products in insects (Scott, 1999).

In many parts of Africa, the malaria vector, *An. gambiae* shows high levels of resistance to pyrethroid insecticides which are the mainstay of vector control (WHO, 2005; 2014; Wanjala et al., 2015; Ranson and Lissenden, 2016). There is evidence that this resistance may reduce the efficacy of treated bednets and indoor residual spraying with pyrethroid based insecticide (N'Guessan *et al.*, 2007; Asidi *et al*., 2012; Ochomo *et al*., 2013; Ochomo *et al*., 2014). In Kenya the frequency of kdr alleles in *An. gambiae* populations has increased over time in western Kenya (Vulule *et al.*, 1994; Stump *et al*., 2004; Mathias *et al*., 2011). Studies carried out on *An. gambiae* and *An. funestus* have shown that gene expression is linked to the detoxification of insecticides changes through the different stages of the mosquito life cycle; these changes in gene expression may have an effect on the relative levels of insecticide resistance at each life stage (Sanders *et al*., 2003; Glunt *et al*., 2011; Christian *et al*., 2011a; Jones *et al.*, 2012).

Studies of resistance mechanisms are key to both understanding the evolution of resistance aimed at mitigating its impact on disease control and to ensure sustainable and efficient vector control. A full understanding is needed not only of the genetic and physiological basis of resistance, but also of the non-genetic factors and other biological processes that influence the expression of detoxification enzymes and the expression of resistance genes other than insecticide exposure in *An. gambiae* that may lead to inefficiency of the control methods. For instance some studies on *An. stephensi* Liston and *Aedes aegypti* (L.) have reported a recent blood meal as one of the factors that may influence insecticide tolerance levels to dichlorodiphenyltrichloroethane ( DDT) and dieldrin (Brown and Pal. , 1971). Studies on *Aedes*

*aegypti* following a murine blood meal have shown expressions of approximately 330 genes that are altered after a blood meal (Sanders *et al*., 2003). The enhanced regulation of cytochrome P450s in response to an avian blood meal has also been identified in *C. pipiens (*Baldridge and Feyereisen, 1989) also the expression of genes have been observed in the midgut of *An.gambiae* after blood feeding (Vlachou *et al*., 2005). Such changes may have an impact on characteristics that affect vector control.

During and after a female mosquito has taken a blood meal a suite of digestive processes are initiated in order to utilize the blood nutrients, with many transcriptional changes peaking after some few hours of feeding (Dana *et al*., 2005; Marinotti *et al*., 2005). The detoxification mechanisms required for the neutralizing harmful components in the *An. gambiae* blood meal may also alter its susceptibility to insecticides probably through increased enzyme regulation. Therefore this study investigated the influence of a blood meal on susceptibility to deltamethrin in *Anopheles gambiae* at different age groups as one of the contributing factors that can lead to inefficiency or ineffectiveness of an insecticide. Mosquito longevity is an important component in malaria transmission (Rajatileka *et al*., 2011). It has been observed that sensitivity to insecticides increases with age (Hodjati and Curtis, 1999; Rajatileka *et al*., 2011; Christian *et al*., 2011a; Jones *et al*., 2012) . This may be assumed to be a function of decreasing enzyme activity due to the reduction of soluble proteins with age (Lines and Nassor, 1991).Therefore in order to understand the impact of insecticide resistance, it is important to consider whether blood feeding promotes resistance in both young and old mosquitoes.

## 1.2 Statement of the problem

Although substantial progress has been made on understanding the causes of pyrethroid based insecticide resistance, few studies have focused on the biological factors that may influence the response to insecticides and the expression of resistance. For instance, during and after a female mosquito has taken a blood meal a suite of digestive processes are initiated in order to utilize the blood nutrients. During the process of blood digestion, the detoxification mechanism required to reduce harmful components in blood may increase the ability of mosquito to tolerate insecticide intoxication through enzyme upregulation. Potentially, an altered insecticide resistance profile may affect on the current control strategies employed by malaria vector control programmes which mostly rely on indoor residual spraying (IRS) and use of insecticide treated nets (ITNS). Hence the current study on determination of the influence of blood meal on susceptibility to pyrethroids in *An.gambiae* as one of the factors that may promote resistance.

## 1.3 General Objective

To determine the influence of blood meal on susceptibility to deltemethrin in *Anopheles gambiae* from Bungoma in Western Kenya.

### 1.3.1 Specific objectives

I. To determine the difference in susceptibility to deltamethrin in *Anopheles gambiae* of Bungoma wild population and Kisumu susceptible strain with different gonotrophic status.

II. To measure metabolic enzyme levels in *Anopheles gambiae* with different gonotrophic status from the two populations.

### 1.3.2 Study Hypothesis

Blood meal does not affect the susceptibility of *Anopheles gambiae* mosquitoes when exposed to deltamethrin insecticide.

## 1.4 Justification of the Study

Since many major biological processes affect gene expression, it is possible that insecticide detoxification gene expression may be stimulated by processes other than insecticide exposure. Hence, it is hypothesized that the enzymes responsible for the detoxification of xenobiotics in the blood meal may influence the subsequent level of susceptibility to insecticide following insecticide exposure.

The Information obtained will be key to both understanding the non-genetic factors contributing to resistance and expression of detoxification enzymes that may lead to inefficiency of the insecticides available and also in developing of insecticide resistant management tools to minimize its impact on disease control interventions.

# CHAPTER TWO: LITERATURE REVIEW

## 2.1 Insecticide Resistance

The development of insecticide resistance has been demonstrated across most insect orders, with an ever increasing prevalence of multiple resistances (Hemingway and Ranson, 2000). The WHO defines insecticide resistance as the "development of an ability in a strain of an organism to tolerate doses of a toxicant which would prove lethal to the majority of individuals in a normal susceptible population of the species"(Zlotkin, 1999). Evolution of insecticide resistance in an insect population arises when there is an increase in the frequency of one or more resistance genes in the population following exposure to insecticides.

Insecticides are classified as either inorganic or organic compounds. WHO recommends the use of organic compounds that are synthetic of origin (WHO, 2004). The organic compounds all belong to four chemical classes:, Pyrethroids (e.g. Deltamethrin,Permethrin and lambdacyathrin) and Organochlorines( e.g. Dichlorodiphenyltrichloroethane (DDT) ) which target voltage-gated sodium channel, Organophosphates (e.g. Malathion) and Carbamates ( e.g. Bendiocarb) which target acetylcholinesterase synapses on the insect neurons(WHO, 2004).

Synthetic pyrethroids i.e. Permethrin, Deltamethrin and Lambdacyhalothrin are the preferred group of insecticides recommended for use in ITNS and IRS owing to their strong insecticidal activity at low concentrations and their low mammalian toxicity (WHO, 2004). The increased resistance of malaria vectors to pyrethroid based insecticides has become a major concern in many countries, and this requires immediate attention because of the limited chemicals available for malaria vector control (WHO, 2004; Santolamazza *et al*., 2008; Awolola *et al*., 2009).

There are two ways through which insects develop resistance to insecticides: the overproduction of detoxifying enzymes that sequester and/or degrade the insecticide before it reaches the nervous system (metabolic resistance). The second mechanism involves mutation of the insecticide target-site which effectively blocks the action of the insecticide. These mechanisms have been studied in various species of insects (Martinez-Torres *et al.*, 1998; Ranson *et al*., 2000; Hemingway *et al*., 2004). Levels of insecticide resistance in field populations of vectors have been shown to vary over relatively small geographical areas and over different seasons (Diabaté *et al*., 2004).

In Kenya studies have shown that both target site insensitivity and metabolic resistance to permethrin are present in *An. gambiae* populations from western Kenya, and that these mechanisms have become more prevalent in response to extensive ITN use (Vulule *et al*., 1999; Ochomo *et al*., 2012; Wanjala *et al*., 2015).

Pyrethroid resistance in most parts of Africa has been partly as a result of in intensive use of insecticides in agriculture (Diabate *et al*., 2002). It has also been observed that the potential of xenobiotics present in polluted mosquito breeding sites affects the mosquito tolerance to pyrethroid based insecticides through cross-induction of particular detoxification genes(Poupurdin *et al*., 2008). This may have negative implications for vector-borne disease control.

### 2.1.1 Impact of Insecticide Resistance on Malaria Control

Insecticide resistance has an impact on the transmission of diseases directly by increasing the number of mosquitoes in the population (Ranson and Lissenden, 2016).Malaria vector control interventions have been hampered by the emergence of insecticide resistance to insecticides used on these interventions (Asidi *et al*., 2012). According to WHO (2014), the malaria vector, *An. gambiae* shows high levels of resistance to pyrethroid insecticides which are the mainstay of

vector control. This has prevented the achievement of malaria eradication and threatens long term ability to control malaria vectors. For instance in South Africa, malaria vector control failure due to metabolic-based resistance to pyrethroid and carbamate insecticide efficacy has been reported (Hargreaves *et al*., 2000).This resistance is closely associated with the presence of a high level of oxidase activity and sometimes conferring cross-resistance to the carbamate insecticide in the local vector *An. funestus* (Brooke *et al*., 2001). This resulted in malaria epidemic in 1999/2000 which was the worst epidemic experienced in South Africa (Hunt *et al*., 2005).

The emergence of phenotypic, genotypic and metabolic resistance to pyrethroid insecticides, and DDT has been reported in Western Kenya, of particular concern was the increase in frequency of the knock-down resistance allele 1014S at the voltage-gated sodium channel in some populations of *An. gambiae s.s.*, (Mathias *et al*., 2011).

The current spread of pyrethroid resistance in the major malaria vectors *An. gambiae* and *An. funestus* emphasizes the need to understand the evolution of insecticide resistance, identify alternative insecticides and for the development and implementation of effective and sustainable resistance management strategies.

### 2.1.2 Insecticide Resistance in Africa

A number of studies have detailed the distribution of insecticide resistance mostly to pyrethroids and DDT in *An. gambiae*: Most studies on the distribution of the L1014F [ West African knock down resistance *(kdr)* mutation] and L1014S (East African *kdr* mutation) mutations in *An. gambiae* populations from Sub-Saharan Africa west of the Rift Valley (Santolamazza *et al*., 2008) indicates that, in *An. gambiae* S-form, the L1014F allele is present in the western area of

Africa i.e. between Senegal and Nigeria with a *kdr* frequency of greater than 50% in most sites. Also, in West – Central area, Cameroon in the North to Angola in the South, extending East wards to Uganda both *kdr* mutations are found in the *An. gambiae* S form. In the M- form of *An. gambiae,* the L1014S allele is absent while the S1014F is present in restricted geographic region in the central part of the Gulf of Guinea i.e. Benin, Nigeria and Cameroon (Santolamazza *et al*., 2008).

*An. funestus* resistance to DDT, pyrethroids and Carbamates was reported from South Africa after the recurrent use of DDT for IRS campaigns in 1950s (Hargreaves et al., 2000). Resistance of *An. funestus* to both DDT and pyrethroid resistance has been detected in Uganda(Verhaeghen *et al*., 2006). Studies conducted in Lower Moshi, Tanzania detected a low frequency of L1014F in *An. arabiensis* population (Kulkarni *et al*., 2006; Matowo *et al*., 2010) while in Uganda, Verhaeghen *et al*. (2006) observed L1014S allele in *An. arabiensis.* In Sudan *An. arabiensis* showed high levels of DDT and permethrin resistance (Matambo *et al*., 2007).

Metabolic resistance has also been observed in some African countries. A study conducted in Chad, indicated that *kdr* mutation was not responsible for the resistance in *An. arabiensis* suggesting alternative mechanisms, probably of metabolic origin are involved (Kerah-Hinzoumbé *et al*., 2008).Brooke *et al*. (2001) Also observed high level of pyrethroid resistance in *An. funestus* populations in southern Mozambique and the main mechanism of pyrethroid resistance was because of elevated levels of one or more monooxygenase enzymes. In South Africa, pyrethroid resistance was detected in *An. funestus* population and linked to elevated levels of monoxygenase activity (Brooke *et al*., 2001).

### 2.1.3 Pyrethroid Insecticide Resistance in Kenya

Two main resistance mechanisms to pyrethroid insecticides have been observed in *An. gambiae s.l.* mosquitoes in Kenya: These are target site mutations (Ranson *et al*., 2000; Stump *et al*., 2004; Kamau *et al*., 2008), and detoxification by elevated enzymatic activity (Vulule *et al*., 1994; Ochomo *et al*., 2013). The first reported case of resistance was in the context of insecticide-treated net use in western Kenya (Vulule *et al*., 1994) where the use of impregnated nets selected for higher oxidase and esterase levels in *An. gambiae* to metabolize permethrin acquired from the nets. Both oxidase and esterase mechanisms could confer cross-resistance to other pyrethroids.

In western Kenya reduced knockdown rates have been seen (Stump *et al*., 2004) the study showed L1014S allele mutation in the *An. gambiae* S form and the *kdr* frequency was about 4% - 8 %, but it was not detected in Coast province. Most recent studies reported a dramatic increase in kdr allele frequency in western Kenya from 1996-2010 (Mathias *et al*., 2011; Ochomo *et al*., 2014; Wanjala *et al*., 2015). This coincided with the scale up of ITNs, and a fixation of kdr L1014S alleles in *A. gambiae s.l* populations (Ochomo *et al*., 2014). Studies by Kamau and Vulule (2006) in central Kenya also indicated that *An. arabiensis* are susceptible to all insecticide. Also Chen *et al*. (2008), observed high monooxygenase levels and kdr allele frequencies in *An. gambiae* and *An. arabiensis* in the western region of Kenya, but not the Great Rift Valley - central region, and the coastal region of Kenya.

The distribution and frequency of these mutations poses serious questions about the sustainability of insecticide-based vector control programs. This is particularly evident when one considers that pyrethroids are the only insecticides recommended by the WHO for insecticide-

treated materials (Weissmann, 2006). Studies of resistance mechanisms are key to understanding resistance to allow designing more finely tuned control strategies that take into account current and historical selection pressures and gene flow patterns

## 2.2 Mechanisms of Insecticide Resistance

Most insecticide resistance mechanisms can be categorized into two major groups, firstly the insensitivity of target site which occurs when the insecticide no longer binds to its target (i.e., insensitivity of the sodium channel, acetylcholinesterase and GABA receptor), or secondly increased metabolic detoxification which occurs when enhanced levels of modified activities of detoxification enzymes prevent the insecticide from reaching its site of action(Vulule *et al*., 1994; Martinez-Torres *et al*., 1998; Hemingway and Ranson, 2000; Hemingway et al., 2004; Amenya *et al.,* 2008). In addition, many insecticides also cause behavioral and physiological changes (Hemingway *et al*., 2004).

### 2.2.1 Insecticide Target Site Insensitivity

Target site resistance is based on alterations of amino acids in the site of action where the insecticide binds, causing the insecticide to be less effective or ineffective at all (Martinez-Torres *et al*., 1998). Altered target sites which translate into insecticide resistance include: the sodium channel gene which is associated with knock down resistance to pyrethroids and DDT, otherwise known as *Kdr* resistance (Martinez-Torres *et al*., 1998; Ranson *et al*., 2000) and acetylcholinesterase gene (AChE1)(Ranson *et al*., 2000; Weill *et al*., 2003) which confers resistance to organophosphates and carbamates and the GABA receptors which confer resistance to the cyclodienes (Hemingway and Ranson, 2000).

Voltage-dependent sodium channel target site insensitivity results from point mutations in the voltage-gated sodium channel gene which is a target site for DDT and pyrethroids on the insects' neurons. Insecticide binding delays the closing of the sodium channel prolonging the action potential and causing repetitive neuron firing, paralysis and eventual death of the insect (Zlotkin, 1999). This mechanism is also termed knock-down resistance in reference to the ability of insects with these alleles to withstand prolonged exposure to insecticides without being 'knocked-down (Davies *et al*., 2007). Two known mutations within the sodium channel gene, also known as *kdr* mutations do occur (Verhaeghen *et al*., 2006). *Kdr* mutation is due to a single nucleotide polymorphism in the gene encoding sub-unit II position 1014 of the sodium channel gene and leads to the substitution of leucine (TTA) for phenylalanine (TTT) at residual L1014F (West African *kdr* mutation). On the other hand, *Kdr* mutation in the same amino acid results in leucine (TTA)-serine (TCA) substitution at the same residual L1014S (East African *kdr* mutation) (Santolamazza *et al*., 2008). The L1014F mutation is the only allele present in West Africa, while L1014S is found in regions both west and east of Africa including Kenya (Santolamazza *et al*., 2008; Mathias *et al*., 2011). While the coexistence of L1014F and L1014S has been reported in several countries in central Africa, L1014F has now been observed in *An. gambiae s.l.* in Kenya (Eric Ochomo pers. comm.).

Acetylcholine is the transmitter at central nervous system synapses in insects. In order for the nervous system to operate properly it is necessary that, once the appropriate message has been passed, excess acetylcholine should be removed from the synapse, both to prevent repetitive firing and to allow a succeeding message to be transmitted (Weill *et al*., 2003). This removal is effected by the enzyme acetylcholinesterase (AChE1), which terminates nerve impulses by hydrolyzing the neurotransmitter acetylcholine. AChE in the insect nerve system is the primary

target of organophosphate (OP) and carbamate insecticides, which inhibit AchE activity by phosphorylating or carbamylating the serine residue within the active site of the enzyme (Weill *et al*., 2003). The insecticide resistance mechanism is due to a single amino acid substitution at position 119 from a glycine to a serine, in the AChE1 catalytic site (G119S) (Djènontin *et al*., 2009). In *An. gambiae*, and other mosquito species, there are two different acetylcholinesterase proteins that are encoded by two different genes and single mutation in the *ace-1* gene explains resistance in *Anopheles gambiae* and *Culex pipiens* (Weill *et al*., 2003).

Resistance to cyclodiene insecticide dieldrin has been associated with mutations occurring in the M2 transmembrane domain of the GABA receptor. Two mutations associated with the dieldrin resistance may occur. The mutation either causes the substitution of alanine 296 to glycine or mutation of the same codon conferring the substitution of alanine to serine (Brooke *et al*., 2006).

### 2.2.2 Metabolic detoxification

The metabolic mechanisms contribute to a decrease in the effective dose available at the target site. It is usually involves over-expression of enzymes capable of detoxifying insecticides or modifications in the amino acid sequences that cause alterations in the levels and activity of detoxifying enzymes(Vulule *et al*., 1999) .The Detoxifying enzymes generally belong to one of three broad classes, namely the mixed-function oxidases(MFO)/ monooxygenases, glutathione S-transferases (GSTs) and non-specific esterases (Hemingway and Ranson, 2000). The insect may produce increased quantities of these enzymes, which either metabolize the insecticide or sequestrate the molecules so they cannot function (Matowo *et al*., 2010). The monooxygenase class are involved in the metabolism of many endogenous (hormones and pheromones) and exogenous (insecticides and plant toxins) (Djouaka *et al*., 2008). P450 Cytochromes are

hemoproteins which act as terminal oxidases in monooxygenase systems. The name originates from its characteristic absorbance peak at 450 nm that appears when these enzymes are reduced and saturated with carbon-monoxide (Nikou *et al*., 2003). A number of P450 studies have suggested that increased levels of insecticide tolerance may be linked to increased levels of CYP4, CYP6, CYP9 and/or CYP12 expression (Scott, 1999).

Pyrethroid resistance due to elevated MFO have been reported in *An. gambiae* (Vulule *et al*., 1999; Nikou *et al*., 2003; Djouaka *et al*., 2008), *An. arabiensis* (Munhenga and Koekemoer, 2011)and *An. funestus* (Brooke *et al*., 2001; Amenya *et al*., 2008) in south Africa. *CYP6Z1,* an adult-specific P450 gene, has been identified from *An. gambiae* associated with pyrethroid reistance (Nikou *et al*., 2003).

Non-specific esterases (NSEs) are commonly involved in the detoxification of organophosphates and carbamates. High levels of these enzymes have also been associated with resistance to permethrin (Vulule *et al*., 1999; Fonseca-González *et al*., 2009; Ochomo *et al*., 2012). Organophosphates and carbamate inhibit B esterases by rapid esterification of the serine residue in the active site, usually followed by a slow hydrolysis of the new ester bond. Therefore, these insecticides can be considered as inhibitors of esterases, because they are poor substrates which have a high affinity for these enzymes (Hemingway and Karunaratne, 1998). NSEs in large amounts causes resistance as the insecticides are rapidly sequestered, even before reaching the target-site of acetylcholinesterase (Hemingway and Karunaratne, 1998). Apart from elevation of the enzyme, amino acid alterations have also been implemented in resistance. An amino acid substitution  within the active site of both house fly and blowfly esterases conferred resistance to organophosphates(Claudianos *et al*., 1999)

The Glutathione-S-transferases (GSTs) are a diverse family of enzymes involved in a wide range of biological processes. They play a central role in detoxification of both endogenous and xenobiotic compounds either directly or by catalysing the secondary metabolism of a vast array of compounds oxidised by P450 cytochromes (Wilce and Parker, 1994; Enayati *et al*., 2005) and are also involved in physiological processes such as intracellular transport, biosynthesis of hormones and protection against oxidative stress (Enayati *et al*., 2005). The enzyme plays an important role in maintaining the redox status of the mosquito cell, particularly in relation to vectorial capacity and resistance (Ranson and Hemingway, 2005). GSTs enzymes metabolise insecticides by facilitating their reductive dehydrochlorination or by conjugation reactions with reduced glutathione to produce water soluble metabolites that are more readily excreted (Wilce and Parker, 1994). They also contribute to the removal of toxic oxygen free radical species produced through the action of pesticides (Enayati *et al*., 2005). Elevated GSTs activity has been implicated in resistance to several classes of insecticides (Ranson *et al*., 2001). In *An. gambiae* elevated GST levels were shown to be associated with DDT resistance (Ranson *et al*., 2001). This GSTs elevation have been observed in Hessian fly and found that the product of the Delta GST genes aid in detoxifying exogenous allelochemicals from the host plant (wheat) (Mittapalli *et al*., 2007).

Metabolic resistance has been identified in vector populations for all major classes of insecticides currently used for vector control, including organophosphate, Carbamates, pyrethroids and DDTs (WHO, 1998). Studies in Tanzania showed that β-esterase mediated hydrolysis and oxidative detoxification by monooxygenases are the predominant mechanisms of permethrin resistance in adult *An. arabiensis* (Matowo *et al.*, 2010). In Kenya studies have

shown metabolic resistance to pyrethroids present in *An. gambiae* populations from western Kenya, and that these mechanism have become more prevalent in response to extensive ITN usage(Vulule *et al*., 1999; Chen *et al.*, 2008; Bonizzoni *et al*., 2012; Ochomo *et al*., 2012).

### 2.3 Factors Influencing Insecticide Resistance

Life stage studies in *An. gambiae* and *An. funestus* have shown that gene expression linked to the detoxification of insecticides changes through the different stages of the mosquito life cycle (Strode *et al*., 2006; Christian *et al*., 2011a). These changes in gene expression could have an effect on the relative levels of insecticide resistance at each life stage (Strode *et al*., 2006). Rajatileka *et al*. (2011) has shown that sensitivity to insecticides increases as mosquitoes age .This may be at least partly explained by an age-related decline in the expression of insecticide detoxification genes (Hodjati and Curtis, 1999; Jones *et al*., 2012).

Some studies have shown that providing mosquitoes with a blood meal may decrease their sensitivity to insecticides (Hunt *et al*., 2005; Spillings *et al*., 2008). During and after a female mosquito has taken a blood meal a suite of digestive processes are initiated in order to utilize the blood nutrients. Sander *et al*., (2003) analysed gene expression within the midgut of *Aedes aegypti* following a murine blood meal. The study showed that the expressions of approximately 330 genes are altered after a blood meal; of particular interest was the increased expression of two P450s whose putative function is detoxification. The up regulation of cytochrome P450s in response to an avian blood meal has also been identified in *C. pipiens* (Baldridge and Feyereisen, 1989*).*

The current spread of pyrethroid resistance in the major malaria vector *An.gambiae* emphasizes the need to understand the factors driving the resistance and expression of detoxification

enzymes other than exposure to insecticides that may lead to inefficiency of the insecticide. Hence, this study on the influence of a blood meal on susceptibility to deltamethrin in *Anopheles gambiae* as one of the controlling factors that can lead to inefficiency or ineffectiveness of an insecticide, may provide more information that can lead to the development and implementation of effective and sustainable resistance management strategies.

# CHAPTER THREE: MATERIALS AND METHODS

## 3.1 Study Site

Mosquito larvae were collected from Bungoma County in Western Kenya ($00^0$35' N, $34^0$35' E; 1386 m a.s.l. (Appendix 1), where previous studies have shown *An. gambiaes s.s* is a predominant species and deltamethrin being the principal insecticide used for vector control in the area (Bonizzoni *et al*., 2012; Ochomo *et al*., 2014; Wanjala *et al*., 2015). The Kisumu strain of *An. gambiae s.s* that is susceptible to pyrethroid insecticides and which has been raised in the Kenya Medical Research Institute- Kisumu, for many years was used as a reference strain(Vulule *et al*., 1994)**.**

## 3.2 Mosquito sampling

Wild *Anopheles gambiae s.l.* mosquito larvae were collected and identified using morphological keys (Gillies and Coetzee, 1987) from a range of their natural breeding sites representative of the diversity of productive *anophelines* development sites in the locality, including puddles, foot and hoof prints on pond margins, shallow wells and tire tracks using the standard 350-ml dippers and hand pipettes thereafter, the aquatic stages were kept in plastic bottles. To avoid the collection of siblings, larvae were sampled from more than one breeding sites, no more than 30 larvae (any instars) were collected per larval development site. The aquatic stages were pooled together then transported to the insectary of the Centre for Global Health Research, Kenya Medical Research Institute (KEMRI) in Kisumu, Western Kenya, and reared under standard conditions (25 ± 2°C; 80% ± 4%: relative humidity with a 12 h: 12 h light/dark cycle.), they were

placed in spring water in small trays and reared on a mixture of tetramin (fish food) and brewer's yeast which was provided daily. Upon pupation, individuals were collected and transferred to cages and allowed to emerge as adults. Emerging adults of Bungoma collections and the Kisumu susceptible strain were both provided with a 10% sugar solution and used for further bioassay tests.

### 3.2.1 Experimental set-up

Emerged female mosquitoes at 2 days old from each of the two groups, namely the wild collected mosquitoes and the susceptible Kisumu strain were separated into 2 cages depending on the number of female mosquito emerged. The female mosquitoes in the first cage were subdivided into 4 groups, three groups were blood fed on arm for 30 minutes and later maintained on 10% sucrose solution. The aim was to generate different gonotrophic stages of mosquitoes namely; blood fed, half-gravid and gravid. WHO bioassay test was carried on this four groups after 5hrs for the blood fed. This time was important because by then the process of digestion would have started. For half gravid mosquitoes they were tested after 24 hrs and gravid after 48 hrs post feeding. The last group was not given a blood meal but only maintained on 10% sucrose and they were considered as unfed group.

The second cage from both population with 2 days old female, were reared under the previous mention insectary and maintained only on 10% sucrose provided daily until when they were 14 days old. The 14 days also underwent the same process as mentioned early with the 2 days old to generate the 4 groups.

The grouping was based on the competence of a female mosquito to transmit the malaria parasite. Malaria, require an extrinsic incubation period in the mosquito of at least 10- 14 days

before the female becomes infective (Rajatileka *et al*., 2011). Hence in order to transmit infection the mosquito must live long enough to pick up the pathogen via a blood meal, survive the extrinsic incubation period and then pass on the pathogen during a subsequent blood meal (Githeko and Ndegwa, 2001). Therefore in order to understand the effects blood meal on insecticide resistance, it is important we consider the older mosquitoes since they are the competent vectors in malaria transmission.

The age of experimental mosquitoes was based on time post-emergence and established the population with only a 2-day range of ages. For instance, if pupae were added to a cage on day 0, the adults in that cage the next day were considered to be one day old. The pupal bowl was then removed from the cage on day 2 when the mosquitoes in the cage were 2 days old.

### 3.3 Insecticide Susceptibility Tests

Bioassays to determine phenotypic resistance was carried out on these groups of mosquitoes with different gonotrophic stages after 5, 24 and 48 hrs post blood feeding mosquitoes. At this time the younger mosquitoes were aged 2-4 days and older mosquitoes 14-16 days. The test was done under ambient room temperature ranging from 25°C to 28°C and relative humidity of 70-80%., WHO impregnated filter papers with 0.05% Deltamethrin were used. Each full set of bioassays were performed with five batches of 25 females from each cohort (field collection and susceptible strain), four test batches (100 mosquitoes) were exposed to insecticide-impregnated filter papers and one test batch (25 mosquitoes) exposed to untreated filter paper which served as a control. The numbers of mosquitoes knocked down was recorded at every 10 minutes intervals during the 1 h-long exposure. After 1-hr exposure to the diagnostic concentration of deltamethrin (0.05%), mosquitoes were transferred to recovery cups and maintained on 10% sucrose solution

provided on cotton pledgets for 24 hrs. Percentage mortality was recorded 24 hours post-exposure for each group. Susceptible mosquitoes after 1 hr exposure and survived mosquitoes after 24hr were immediately collected and preserved at -20°C prior to metabolic enzyme analysis. Here we defined "resistant" as the mosquitoes that were alive 24 hours after 60-min exposure to the insecticides in the WHO tube bioassay, and "susceptible" as the mosquitoes that were knocked down within the 24-hr recovery period. Mosquitoes were considered Knocked down if they were motionless, even when they were mechanically stimulated. This is reasonable because motionless mosquitoes after bioassay exposure rarely recovered and survived (WHO, 2013).

**3.4 Enzyme Assays**

Expression of detoxifying enzymes was measured using microplate enzyme system to quantify the levels of detoxifying enzymes (Oxidases, glutathione S transferase and non-specific esterases) in individual mosquitoes as described in Brogdon *et al.* (1988). To ensure that the presence of blood could not interfere with the biochemical assays, the abdomen of the individual mosquitoes was removed (Verhaeghen *et al*., 2009; Ranson and Lissenden, 2016). The remaining head-thorax portion was homogenized in 200 µL of 0.05 M potassium phosphate buffer, pH 7.2, and diluted to 1.5 mL with 0.05 M potassium phosphate buffer. For measurement of esterase activity, the following protocol was conducted in 96-well microtitre plates of 300 µL. To each 100 µL of homogenate, a 100-µL aliquot of *β*-naphthyl acetate (56 mg/10 mL acetone with 90 mL buffer) was added and the preparation was incubated at room temperature for 10 min. A 100-µL aliquot of dianisidine (100 mg/100 mL water) was then added. Absorbances of samples were read with a plate reader at 540 nm.

For oxidase activity, 100 µL of mosquito homogenate was transferred into wells of microtitre plates and 200 µL of 3,3_5,5_-tetramethyl-benzidine dihydrochloride hydrate (50 mg/ 25 mL methanol/75 mL 0.25 M Sodium acetate buffer) was added. A volume of 25 µL of 3% hydrogen peroxide was then added to each well and incubated for 5 min. Sample absorbance was read at 620 nm.

For quantification of glutathione-S-transferase (GST) activity, aliquots (100 µL) of the mosquito homogenate sample were transferred to wells of microtitre plates, and 100 µL of reduced glutathione (61 mg/100 mL KPO4 buffer) was added per well. Next, 100 µL of 1-chloro-2,4-dinitrobenzene (cDNB) (20 mg/10 mL acetone in 90 mL KPO4 buffer) was added and absorbance read immediately at 340 nm. Absorbance was recorded again at 5 min and the difference in absorbance between the two times per sample was determined.

To normalize enzyme activity readings, total protein of samples was determined as previously described in Brogdon *et al.* (1988). A 20-µL aliquot of mosquito homogenate was transferred into wells of microtitre plates and 80 µL KPO4 buffer added. A volume of 200 µL of protein dye reagent 20 mL Bio-Rad protein dye concentrate/80 mL distilled water (dH2O) was then added and optical densities read immediately at 620 nm. For all enzyme assays, each sample was tested in triplicate.

**3.5 Data Analysis**

Data collected was entered into excel spreadsheet, results of the 24-h WHO bioassays were analyzed based on WHO criteria, where the mortality was calculated as a percentage of individuals that died within 24 hours of exposure: mortality <90% = confirmed resistance; 90-97% = possible resistance (with presence of resistant genes to be confirmed); 98-100% = susceptible (WHO, 2013). The mean percentage mortality was calculated for each group and the

percentage mortality graphs for each cohort generated using Graphpad prism version 5.02 software. All means were compared using either a 2-sample t-test or a 1-way Analysis of Variance (ANOVA) with a Tukey comparison-of-means as a post-hoc test. All confidence intervals were set at 95%.Comparisons between the groups (unfed, fed, half gravid and gravid) for each *An. gambiae* population were based on 1-way Analysis of Variance (ANOVA. In order to determine significant differences in enzymatic expression in each population, absorbance values were compared between the knocked down and live individuals from the groups using analysis of variance with three groups: the first group were individuals that survived exposure, second group were individuals that died during exposure and third group were live, unexposed individuals. Similarly, absorbance values were compared between insecticide-exposed and unexposed, Kisumu strain *An. gambiae s.s.* females. Statistical analyses were performed using SPSS Version 21.

### 3.5 Ethical Considerations

The study proposal was reviewed and approved by Graduate School Board of Maseno University (Appendix II). This study was part of a major study titled "Malaria vector surveillance in the context of enhanced malaria control in Western Kenya" which had approval, SSC No.2776: cleared by KEMRI/ National Ethical Review Committee (Appendix III).For mosquito collection, chief and village elders from the area were briefed on the study and permission requested to carry out the sample collection in their area, oral consent was obtained from field owners in each location. These locations were not protected land, and the field studies did not involve endangered or protected species. The study participants were consented before participating in the study by filling a consent form (Appendix IV)

# CHAPTER FOUR: RESULTS

## 4.1 Insecticide Susceptibility Tests

In the WHO tube bioassays, 2-4 day old unfed *An. gambiae s.s.* from field collections had mortality of 83% while the older females aged 14-16 days showed 98% mortality which was significantly different ( 2 sample t-test: $p < 0.047$; $t = -2.176$) ( Fig. 2). The fed groups aged 2-4 days female had mortality rate of 37%, 36% and 60 % for blood fed, half gravid and gravid respectively. The older females (14-16 days) had mortality rates of 86%, 85% and 88% for blood fed, half gravid and gravid respectively. The mortality rates observed were lower than those obtained when these females from both age groups were unfed (83% for 2-4 days and 98% for 14-16 days old; Table 1). The Kisumu strain of *An. gambiae s.s* was 100% susceptible to deltamethrin independent of their abdominal status (Table 1).

The 2-4 days old fed (blood fed half gravid and gravid) wild females showed less than 50% knockdown after one hour exposure to deltamethrin (0.05%) compared to unfed counterparts that showed 50% knock down after 50 minutes of exposure. For 14-16 days old fed females, showed 50% knockdown after 60 minutes of exposure unlike the unfed females which took 20 minutes to obtain the same (Fig. 3). Comparing the mortality within the mosquito age groups using one way ANOVA there was a significant difference between the unfed and fed groups ( blood fed, half gravid and gravid) in susceptibility to deltamethrin (1-Way ANOVA: $p < 0.046$; $F = 2.568$, $df = 3$) ( 2-4 days old ) and $p < 0.047$; $F = 4.027$, $df = 3$ (14-16 days old). However, the mortality rate for gravid females aged 14-16 days old from Bungoma was not significantly different from the unfed females ($P > 0.05$; $F = 2.568$, $df = 3$).

With the susceptible Kisumu strain of *An. gambiae s.s* there was a difference in knockdown rate with more than 50% of unfed mosquitoes from both age groups, knocked down within the first 20 minutes as compared with fed groups (blood fed, half gravid and gravid) , which took about 40 minutes to obtain 50%, knock down (Fig.4). There was no significant difference between the unfed and fed groups (blood fed half gravid and gravid) in susceptibility to deltametrin with this population (1- way ANOVA: P >0.05).

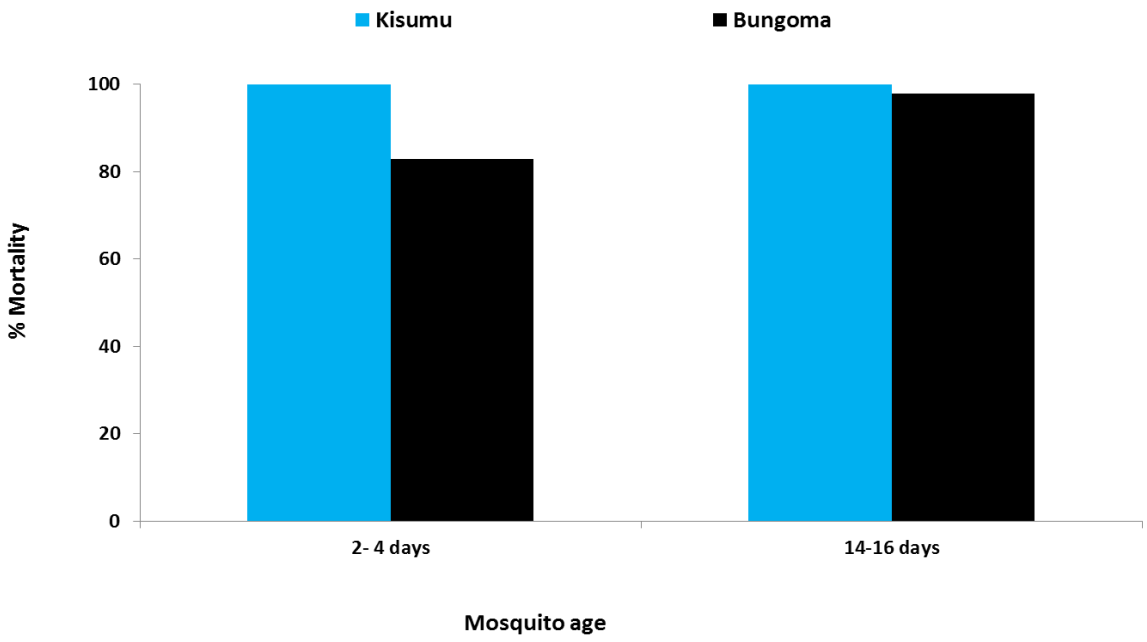


**Figure 1.** Percentage mortality for field caught female *Anopheles gambiae* mosquitoes (Bungoma) and susceptible *Anopheles gambiae s.s* mosquitoes ( Kisumu strain ) exposed to Deltamethrin.

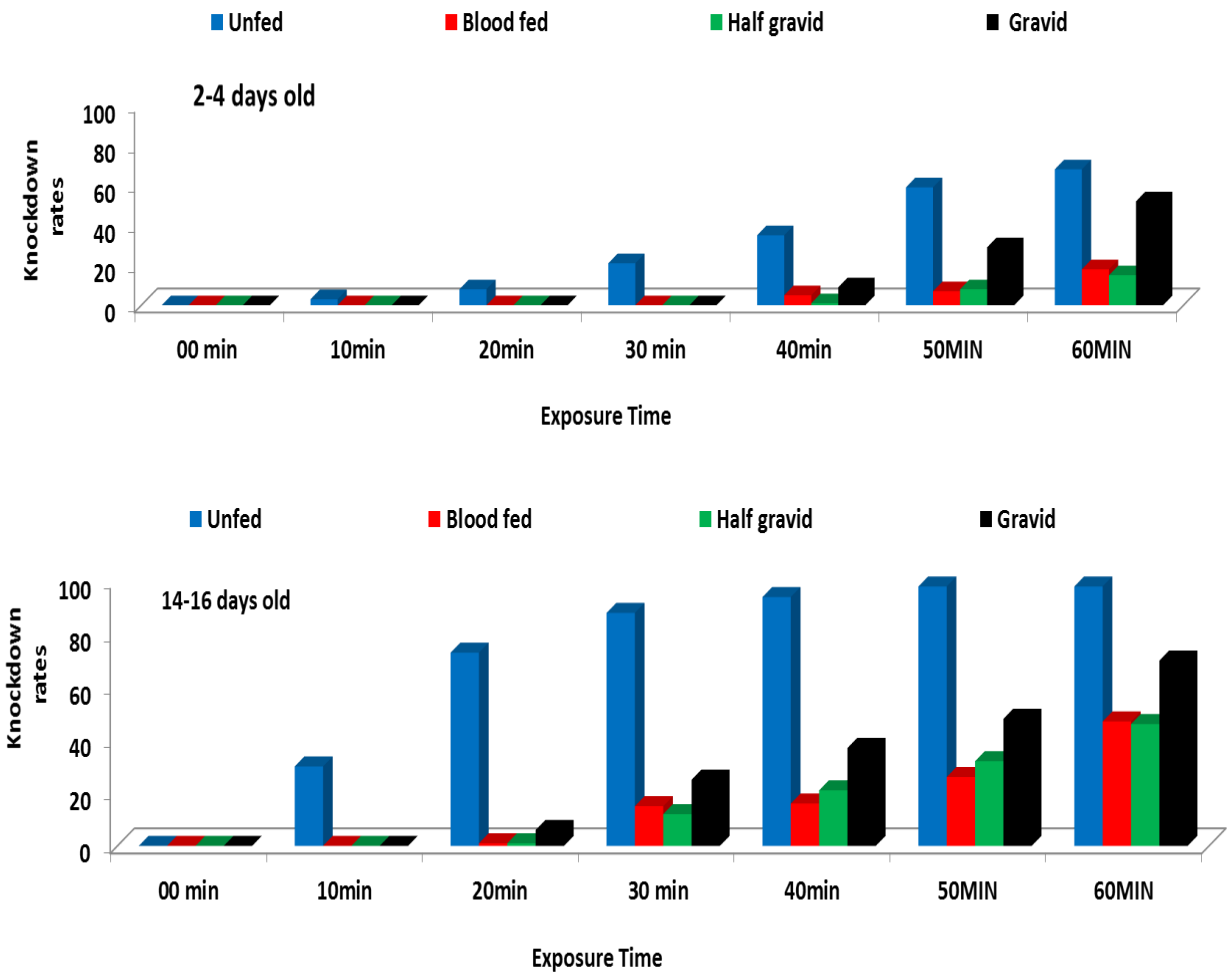


**Figure 2:** Knockdown rate at different time intervals of *Anopheles gambiae s.s* Bungoma population with different gonotrophic status exposed to Deltametrin **(**0.05%) for 1 hour.

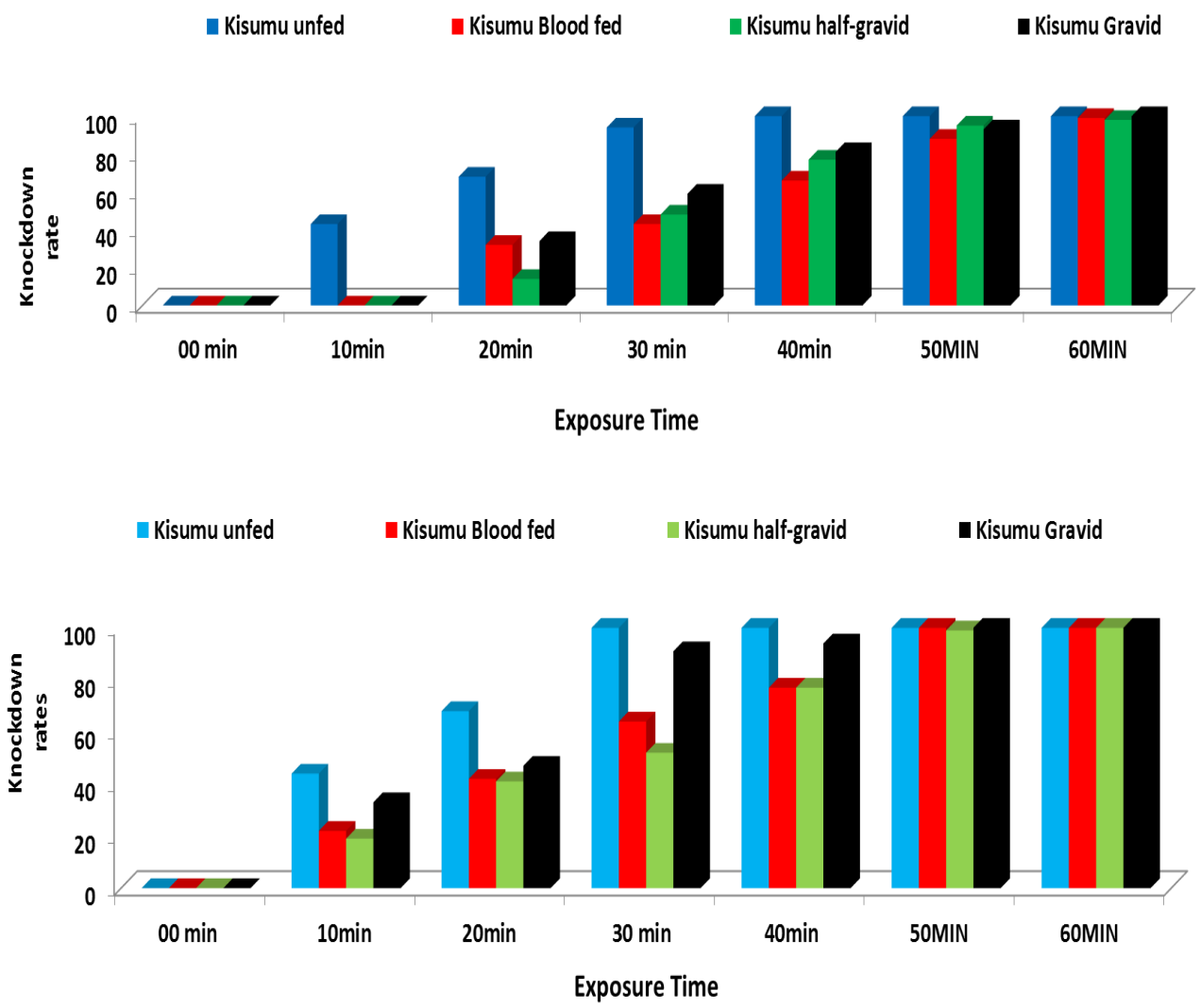


**Figure 3:** Knockdown rate at different time intervals of *Anopheles gambiae s.s* Kisumu population (susceptible) with different gonotrophic status exposed to Deltametrin **(**0.05%) for 1 hour.

**Table 1:** Comparative susceptibility of female *An. gambiae* populations to deltamethrin (0.05%) regarding their age and abdominal status (1): Susceptible >98%; (2): Resistance suspected 90-97%; (3): Resistant < 90% (WHO, 2013).

| Locality | Age | Abdominal status | Number tested | Mortality % | WHO Criteria |
|---|---|---|---|---|---|
| **Bungoma** | **2-4 days old.** | **Unfed** | **100** | **83%** | **Resistant** |
| | | **Freshly fed** | **100** | **37%** | **Resistant** |
| | | **Half gravid** | **100** | **36%** | **Resistant** |
| | | **Gravid** | **100** | **60%** | **Resistant** |
| **Bungoma** | **14-16 days old.** | **Unfed** | **100** | **98%** | **susceptible** |
| | | **Freshly fed** | **100** | **86%** | **Resistant** |
| | | **Half gravid** | **100** | **85%** | **Resistant** |
| | | **Gravid** | **100** | **88%** | **Resistant** |
| **Kisumu strain 100% susceptible independent of gonotrophic status** | | | | | |

### 4.2 Enzymes Activities

Both young and old wild caught mosquitoes that survived exposure to deltamethrin had higher oxidase enzyme activity levels compared with those that were knocked down during exposure and those that had not been exposed ($F_{11,466}$ = 53.648, P < 0.001 for 2-4 days old and $F_{11,360}$=50.648, P<0.001 for 14-15 days old (Fig. 5). The survived fed group had higher oxidase

activity levels compared to survived unfed group from both age groups ($F_{11,466}$ = 53.648, P < 0.001 for 2-5 days old) and ( $F_{11,360}$ =50.648, P < 0.001 for 14-15 days old; Fig. 5).

For non-specific esterases enzyme levels, unexposed live and exposed knocked down mosquitoes from both age groups showed a lower enzyme activity levels as compared to the survived exposed individuals ($F_{11,466}$ =87.648 P< 0.001 for 2-5 days old and $F_{11,360}$=19.636, P<0.001 for 14-15 days old; Fig. 6). The young (2-5 days old) unfed survived group showed a significant decline in enzyme levels when compared to the fed survived individuals (blood fed andhalf gravid) ($F_{11,466}$ =87.648 P< 0.001; Fig.6). For 14-15 days old, the fed survived individuals showed higher enzyme elevations as compared to unfed survived individuals, though it was not statistically significant ($F_{11,360}$=19.636, P> 0.05;Fig.6).

For Glutathione S-transferases ( GSTs) enzyme activity, in both the young and old mosquitoes, those that survived exposure to deltamethrin had higher enzyme activity levels compared with mosquitoes that were knocked down during exposure and with mosquitoes that had not been exposed ($F_{11,466}$=84.286, P<0.001 for 2-5 days old and $F_{11,360}$=20.737, P<0.001 for 14-15 days old). Survived fed group in the 2-5 days old mosquitoes had a higher enzyme elevations compared to the survived unfed individuals ($F_{11,466}$=84.286, P<0.001; Fig. 7).

Comparison of the enzyme activity in 14-15 days old survived unfed mosquitoes with the survived fed groups (blood fed and half gravid), revealed that the fed group showed high elevations which was statistically significant than the unfed ones. ($F_{11,360}$=20.737, P<0.011; Fig. 7).

For the Kisumu susceptible strain, oxidases , non-specific esterases and GSTs levels were higher in both younger (2-5 days old) and older (14-15 days old) fed groups compared to the unfed groups (P<0.05). There was no significant difference in elevation of all the three enzyme

measured between the means of unexposed live groups and the exposed knocked down groups for both age groups (P>0.05).

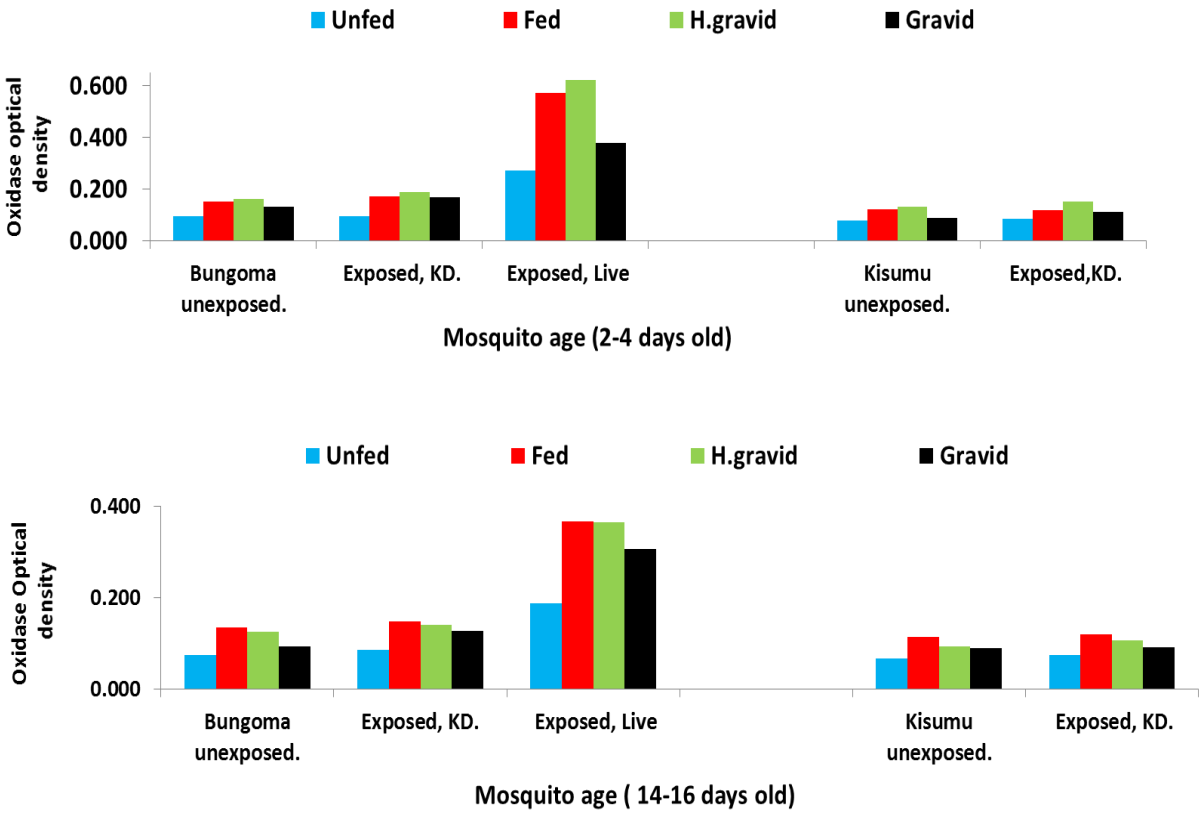


**Figure 4:** Oxidase enzyme activity measured in biochemical assays in female *Anopheles gambiae s.s.* with different gonothrophic status from Bungoma and Kisumu population

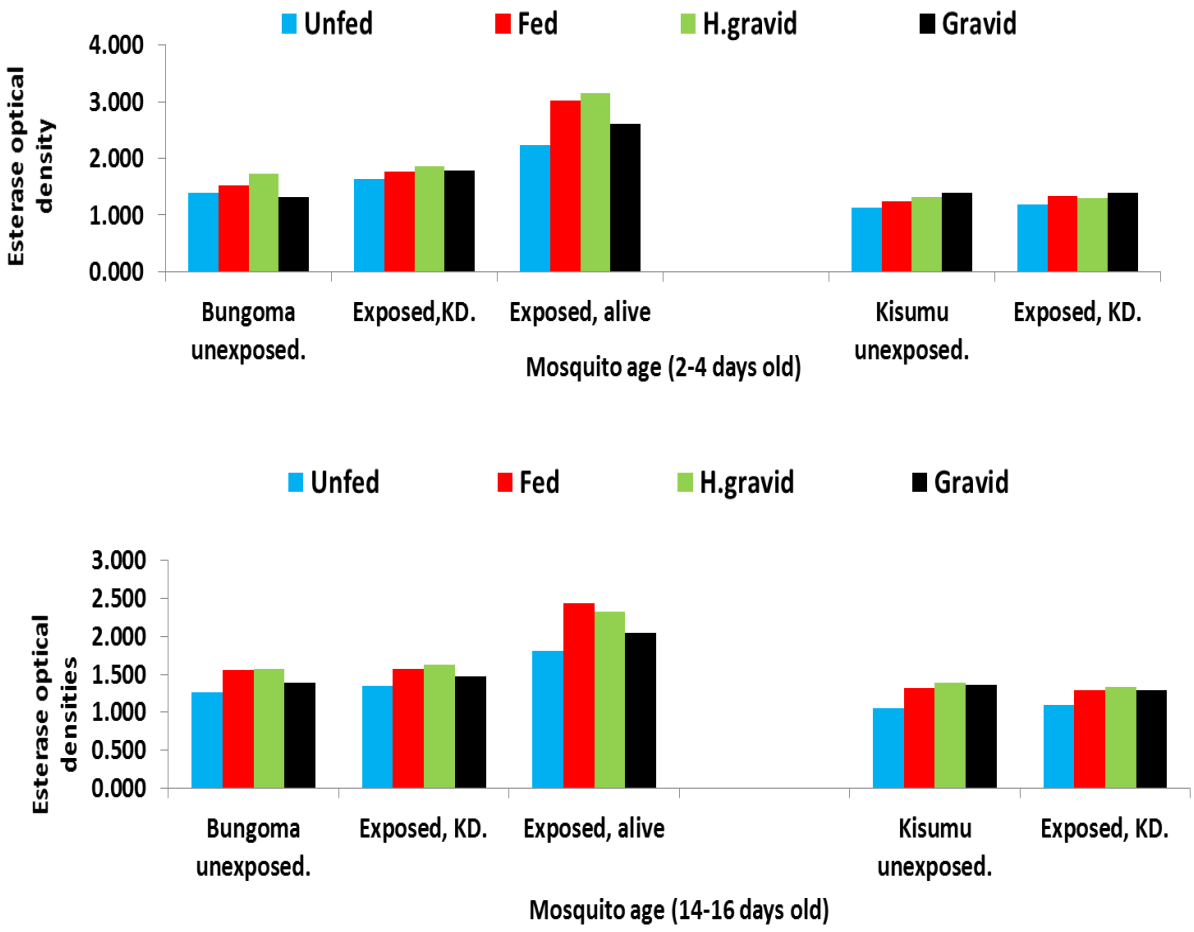


**Figure 5**: Showing enzyme activity (Non-specific esterases) measured in biochemical assays in Field caught *Anopheles gambiae* Bungoma population female and Kisumu strain adults with different gonotrophic status.

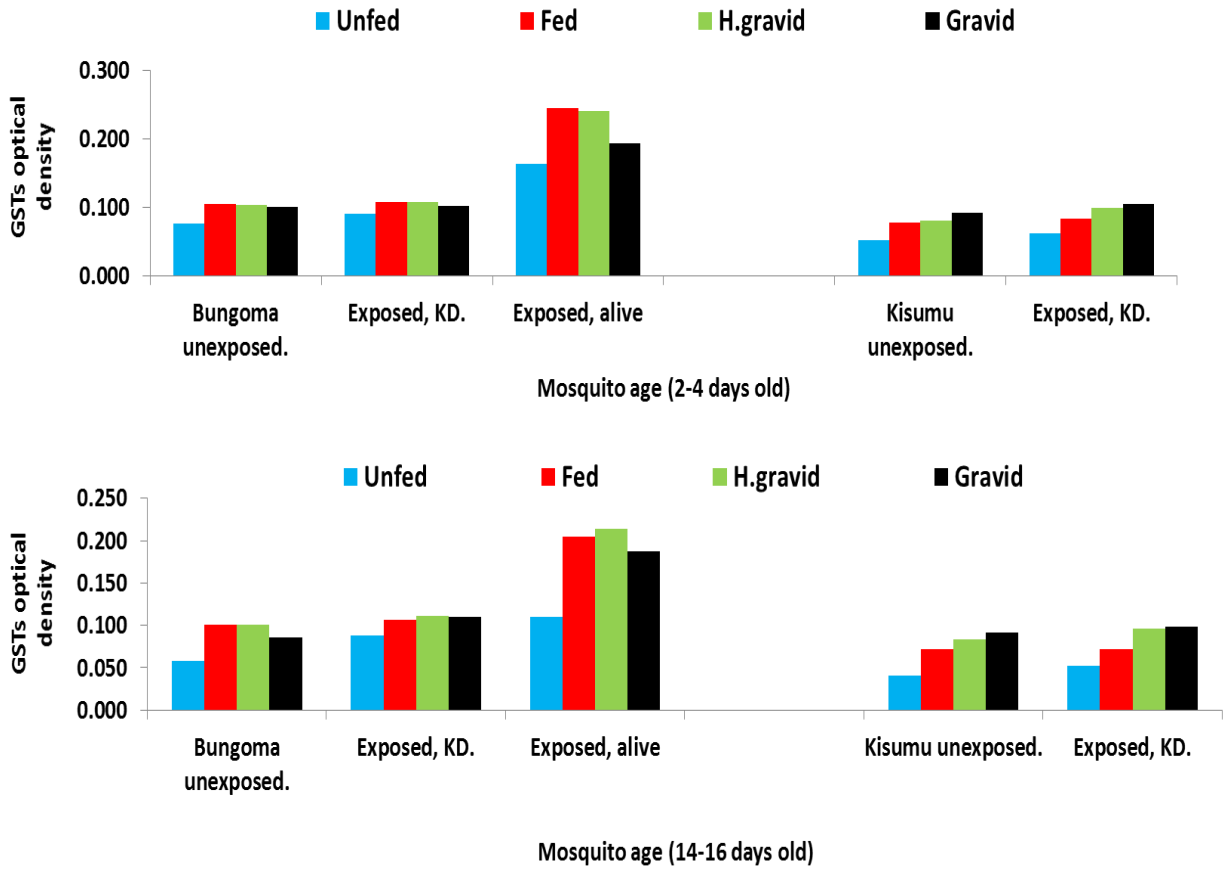


**Figure 6:** Showing activity of Glutathione s transferase enzyme measured in biochemical assays in susceptible *Anopheles gambiae s.s* Bungoma and Kisumu female adults with different gonotrophic status.

# CHAPTER FIVE: DISCUSSION

Insects may survive the toxic effect of insecticides by different physiological mechanisms including target-site insensitivity and elevated detoxifying enzyme production (Martinez-Torres *et al.*, 1998; Enayati *et al*., 2005). In Western Kenya, pyrethroid resistance in *An. gambiae* is likely mediated by a combination of *kdr* and metabolic mechanisms as the primary mode of resistance (Mathias *et al*., 2011; Bonizzoni *et al*., 2012; Ochomo *et al.*, 2013). This study investigated the influence of blood meal on susceptibility to deltamethrin in *Anopheles gambiae* as one of the contributing factor that can lead to ineffectiveness of an insecticide.

Mortality rates were lower in the blood fed, half gravid and gravid mosquitoes in comparison to the unfed mosquitoes, indicating that pyrethroid susceptibility is affected by the abdominal status of the mosquitoes. A similar phenomena was observed in South Africa where tolerance of female *Anopheles funestus* to pyrethroids increased about two-fold at 24 h after a blood meal (Spillings *et al*., 2008). In addition, similar results were observed on bed bugs *Cimex lectularius* L. where those fed recently on a blood meal survived significantly longer compared with the unfed ones on their exposure to fresh or aged residual deposits of chlorfenapyr and aged residual deposits of deltamethrin (Choe and Campbell, 2014). The low mortality rate of wild population may also suggest the additive effects of target site mutations and metabolic detoxification as this has been shown in different studies carried out from these area (Mathias *et al*., 2011; Ochomo *et al*., 2012; Bonizzoni *et al*., 2015; Wanjala *et al*., 2015).

The study found that the older unfed females (14-16 days old) mosquitoes which form that proportion of the population actively transmitting malaria, showed reduced phenotypic resistance compared to the younger 2-4 day old mosquitoes when exposed to deltamethrin. The results were

similar to previous studies on *Anopheles gambiae* that have shown that sensitivity to insecticides increases as mosquitos age (Hunt *et al*., 2005; Rajatileka *et al*., 2011; Jones *et al*., 2012). Another observation on *An. stephensi* and *An. gambiae* showed a reduction in mean knockdown times of 10 day old adults, relative to newly emerged mosquitoes (Hodjati and Curtis, 1999). However, a converse situation was observed in the blood fed, half gravid and gravid older females, where there was a decline in mortality rate compared with the unfed group of the same age; this indicated that the presence of blood meal reduced the effect of the insecticide on the mosquitoes especially as the mosquitoes aged.

The increase in insecticide susceptibility in these unfed field collected mosquitoes that showed high phenotypic resistance in WHO susceptibility tests at younger age, might be a trade-off between energy used for defense against insecticides, onset of senescence and also increase in the rate of cuticle permeability (Jones *et al*., 2012). The reduction of soluble proteins with age (Hodjati and Curtis, 1999) may also explain the difference in enzyme levels between the younger 2-4 days old and older 14-16 days old females from this same population.

Acquisition of blood meal for reproduction by female mosquitos involves a complex series of biological events which may present metabolic changes and induce oxidative stress (Dana *et al*., 2005). The release of reactive oxygen species during the digestion of blood meal (heme) can be toxic to mosquitoes without detoxification enzymes. Multiple amino peptidase isolated from the midgut of blood feeding insects have been linked to play a role during blood digestion, this has also been associated with the increase in detoxification enzymes involved in metabolic cycle for the transport of certain amino acids across the membrane of the malpighian tubules (Enayati *et al.*, 2005) Therefore, detoxification enzymes would be elevated so as to enable the female mosquito to cope with the metabolic challenge induced during blood ingestion (Strode *et al*.,

2006). These could have an effect on the relative levels of insecticide resistance. The current study clearly suggests the differential effect of deltamethrin to female mosquitoes of Bungoma population with different gonotrophic status. The increase in mortality with age in the unfed group suggests that without increased enzyme activity induced by blood meal other mechanisms involved may become less efficient with age. Similar effects were observed in *Anopheles arabiensis,* where blood meal induced a variable effect on mortality, with the younger and older blood fed females showing significantly reduced mortality to deltamethrin (Oliver and Brooke., 2014).

This study demonstrates variability in enzyme elevations between the unfed and fed groups. This concurs with previous studies in South Africa that showed upregulation of some of cytochrome P450 genes in association with blood feeding in *An. funestus*, the gene that has been linked to overproduction of detoxification enzyme oxidase (Spillings *et al*., 2008). Another study carried out on *Aedes aegypti* also observed expressions of approximately 330 genes are altered after a blood meal and that the genes spanned a broad spectrum of processes, including nutrient uptake and metabolism and stress responses. Of particular interest was the increased expression of two cytochrome P450s whose possible function is detoxification (Sanders *et al*., 2003). The current study observed significant high elevation of oxidase enzyme activity in the blood fed, half gravid and gravid groups when compared to unfed females aged 2-5 days old. Similar situation was observed in 14-15 days old females of the same population. These findings are in part, similar to previous studies on field collected *An. minimus s.s*. population with different physiological status showing relatively high levels of P450 monooxygenases (Verhaeghen *et al*., 2009).

GSTs and non-specific esterases were equally elevated in the fed groups when compared to unfed groups for both age groups. Some studies have shown high levels of non-specific

esterases in younger and older blood fed *An. funestus* compared to unfed counteparts (Oliver and Brooke., 2014). GSTs have been shown to have antioxidant properties and function in response to oxidative stress (Vontas *et a*l., 2001). Since some GSTs have peroxidase activity, high elevations in their expression may reduce the oxidative stress by the removal of reactive oxygen species associated with the digestion of the blood meal and subsequent increase in insecticide tolerance (Vontas *et al*., 2001). Increased metabolic activity during the process of blood digestion and oogenesis has been shown to increase the $H_2O_2$ levels in hemolymph, which in turn is associated with high levels of GSTs (Dejong *et al*., 2007). This may speculate the higher GSTs activity in blood fed and half gravid female mosquitoes observed in this study, since it has been shown that high levels of GSTs are associated with the enhanced levels of $H_2O_2$ as GST scavenges $H_2O_2$ (Dejong *et al*., 2007). Previous studies on *An. funestus* demonstrated that blood fed *An. funestus* displayed elevated delta-class GST expression genes linked to high levels of GSTs enzyme activities which play a role in insecticide detoxification (Spillings *et al*., 2008), multiple blood-feeding was also reported to exert a significant enough effect on the regulation of those detoxification enzymes associated with insecticide resistance to maintain the expression of resistance in aging *An. arabiensis* females in south Africa (Oliver and Brooke., 2014). Other blood feeding arthropods, including *Rhodnius prolixus*, also have been shown to upregulate detoxification gene expression following a blood meal, likely as a response to the large quantities of hemoglobin that could otherwise be toxic(Paes *et al*., 2001**;** Graca-Souza *et al*., 2006). It is possible that there is some cross activity between enzymes involved in blood digestion and those involved in insecticide detoxification as observed in insecticide-resistant *An. funestus*, meaning the same enzymes used to digest blood meals may also be used to metabolize insecticides (Spillings *et al.*, 2008)

# CHAPTER SIX: CONCLUSION AND RECOMMENDATION

## 6.1 Summary of Results

This study observed changes in susceptibility following exposure to deltamethrin that occur during the gonotrophic cycle of female *Anopheles gambiae*: 2-4 days old females showed decreased susceptibility shortly after blood feeding. The 14-16 days old blood fed and gravid females showed a decline in mortality when compared with unfed group of the same age. Th enzyme level was equally elevated on the blood fed, half gravid and gravid mosquitoes compared to unfed counterparts. In view of the primary measure of successful vector control in the reduction of transmission risk and disease incidence by quantitatively reducing vector densities that feed and rests indoor, the present data suggest that blood meal combined with already effective detoxification mechanism will enhance the expression of phenotypic resistance and also reduce the susceptibility of older female mosquitoes which actively transmit malaria.

## 6.2 Conclusion

These findings provide additional evidence of the effects of blood feeding in *An. gambiae* susceptibility following exposure to deltamethrin and it suggests that, selective mortality would favor individuals that had taken blood 1 or 2 days before exposure to deltamethrin. The observations of insecticide resistance in older blood fed individual indicates that tolerance could be developed over time due to blood feeding and in this regard, blood feeding would be seen to potentiate female Anopheles mosquitoes to seek and find blood meals despite the use of insecticide based interventions. The higher oxidase, non-specific esterases and GSTs enzymes activity observed could explain why the fed groups of *An. gambiae s.s* Bungoma population

exhibited high phenotypic resistance to deltamethrin as compared to when they were unfed. These results were partly similar to previous studies on *An. arabiensis* where blood-feeding was observed to affect the regulation of those detoxification enzymes also associated with insecticide resistance to maintain the expression of resistance in aging females, or at least to reduce the decline in resistance expression with age.

### 6.2.1 Recommendations

To improve vector management tools, much understanding of how resistance develops in field populations is needed. Many factors influence vector susceptibility to insecticide. Among these factors, there are physiological status and mosquito age. Therefore this study recommends:

1. Gonotrophic status of vector population to be considered when designing adult mosquito control intervention.
2. The inclusion of 2-3 days blood fed younger female mosquitoes during routine insecticide susceptibility on the field populations. This is important because blood-feeding may enhance the expression of resistance to a level where it is detectable using the WHO bioassay system, whereas testing non blood fed females only could allow newly emerging resistance to go undetected for a longer period, facilitating the spread of resistance alleles through affected populations.

### 6.2.2 Recommendations for future studies

1. Further analysis on the specific expressed genes linked to detoxification enzymes after the female vectors have taken a blood meal should be investigated and the operational impact should be further studied, as it may have implications for the sustained efficacy of

the indoor residual spraying based control programmes that targets those female mosquitoes that have taken a blood meal and are resting indoors.

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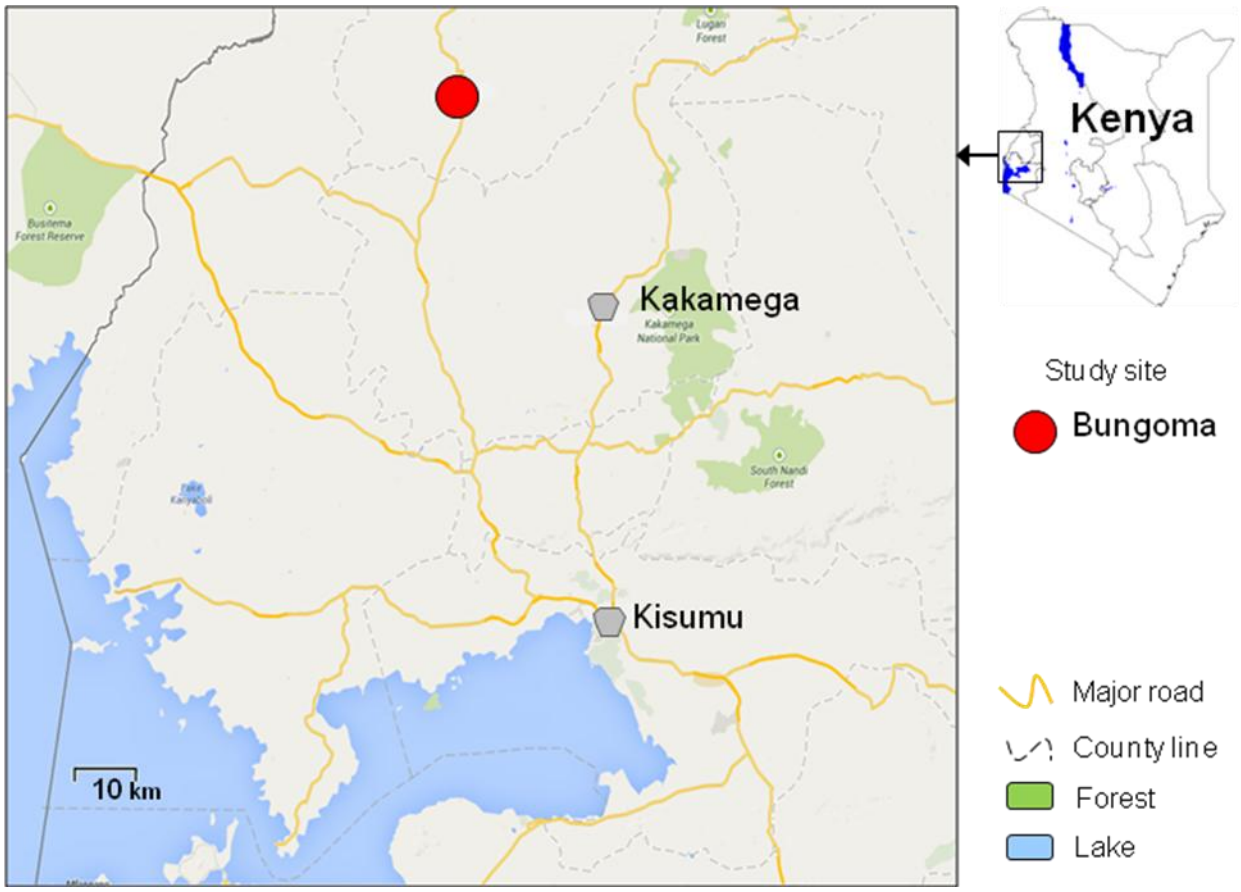
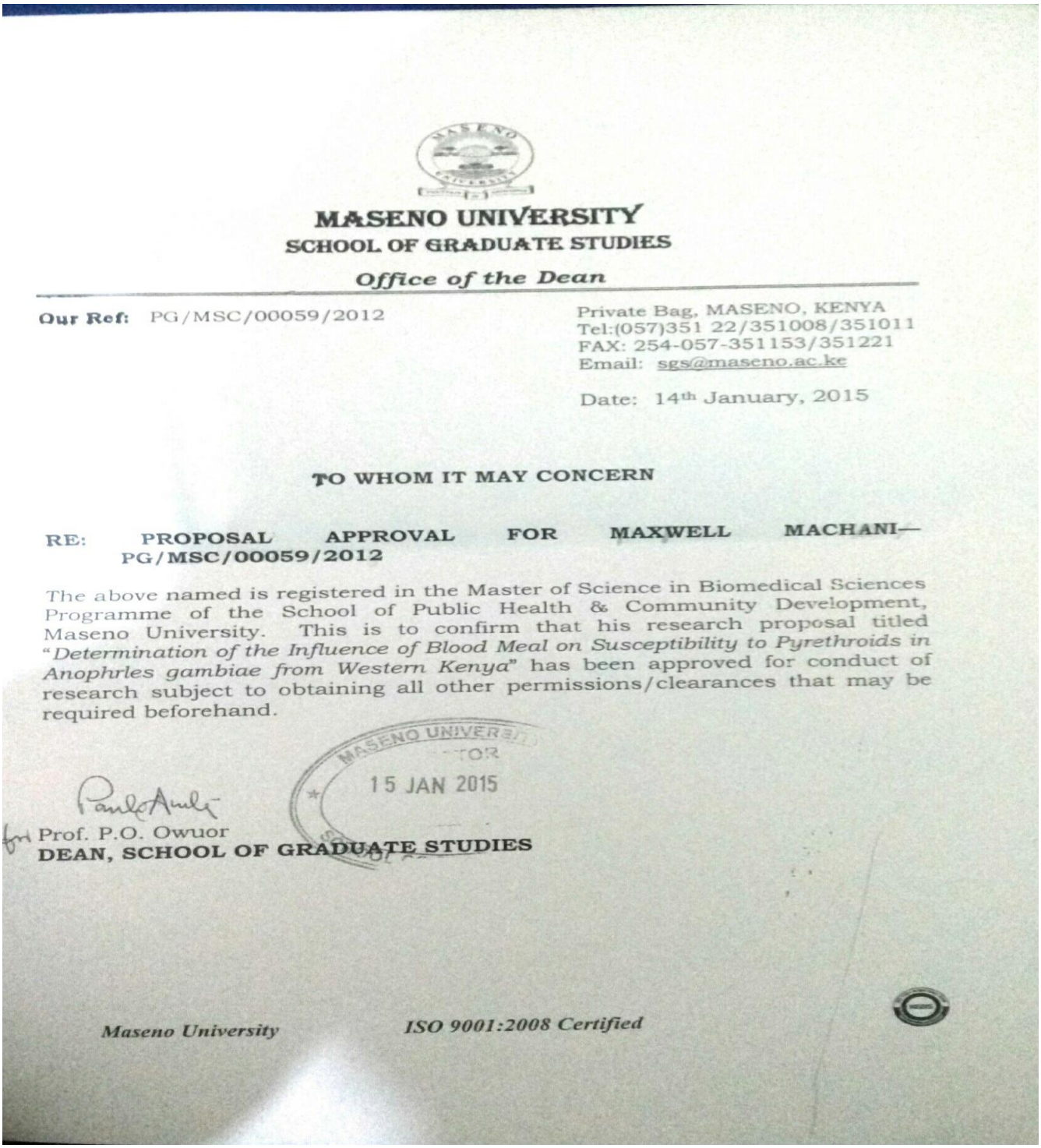
**APPENDICES.**

Appendix I: A map of Kenya showing mosquito sampling sites



**Appendix II**: Study approval by graduate school.



**MASENO UNIVERSITY**
**SCHOOL OF GRADUATE STUDIES**
***Office of the Dean***

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Date: 14th January, 2015

**TO WHOM IT MAY CONCERN**

**RE: PROPOSAL APPROVAL FOR MAXWELL MACHANI— PG/MSC/00059/2012**

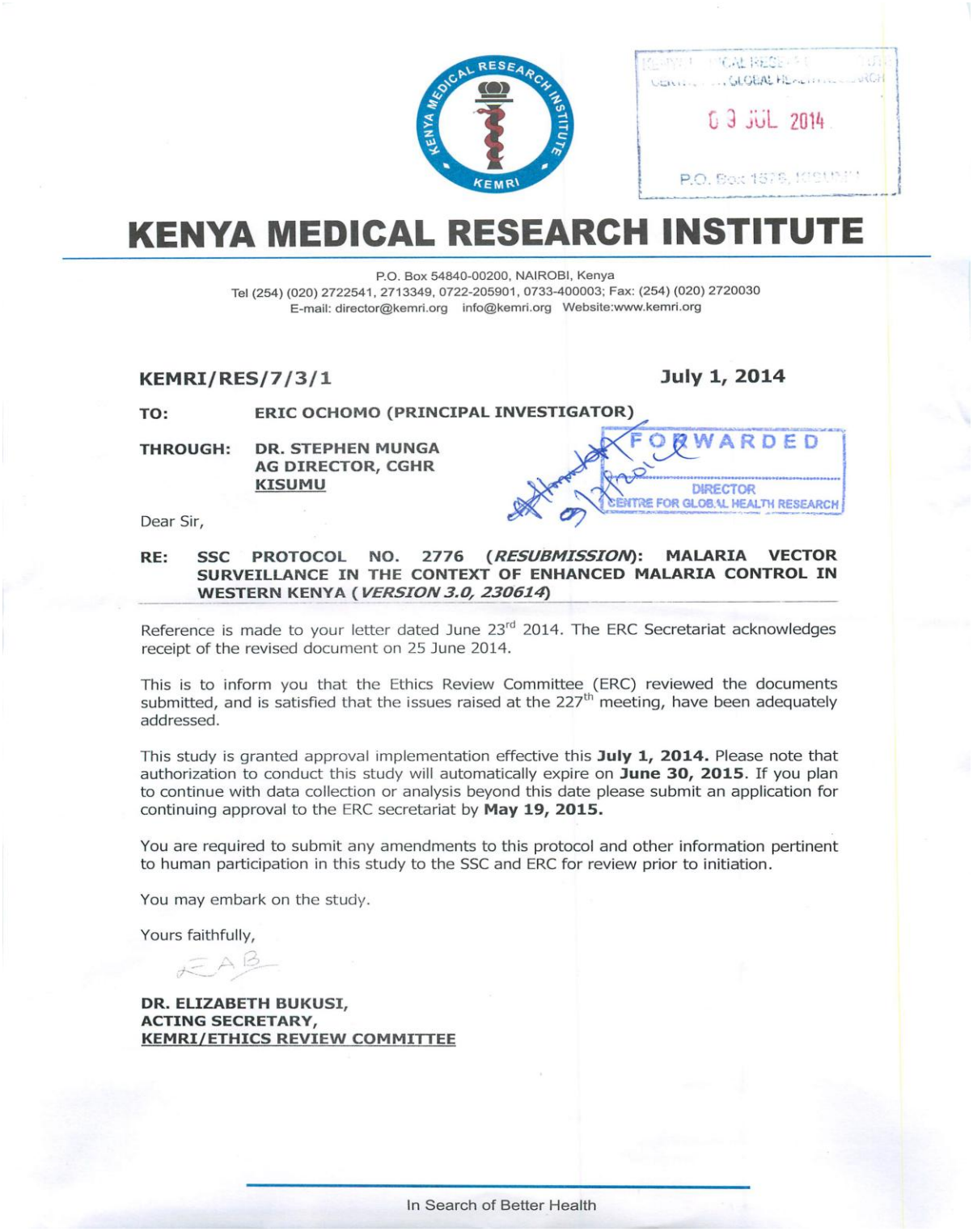
The above named is registered in the Master of Science in Biomedical Sciences Programme of the School of Public Health & Community Development, Maseno University. This is to confirm that his research proposal titled "*Determination of the Influence of Blood Meal on Susceptibility to Pyrethroids in Anophrles gambiae from Western Kenya*" has been approved for conduct of research subject to obtaining all other permissions/clearances that may be required beforehand.

MASENO UNIVERSITY
15 JAN 2015

for Prof. P.O. Owuor
**DEAN, SCHOOL OF GRADUATE STUDIES**

*Maseno University* *ISO 9001:2008 Certified*

**Appendix III**: Study approvals from KEMRI



KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

**KEMRI/RES/7/3/1** **July 1, 2014**

**TO:** **ERIC OCHOMO (PRINCIPAL INVESTIGATOR)**

**THROUGH:** **DR. STEPHEN MUNGA**
**AG DIRECTOR, CGHR**
**KISUMU**

FORWARDED
DIRECTOR
CENTRE FOR GLOBAL HEALTH RESEARCH

Dear Sir,

**RE: SSC PROTOCOL NO. 2776 (*RESUBMISSION*): MALARIA VECTOR SURVEILLANCE IN THE CONTEXT OF ENHANCED MALARIA CONTROL IN WESTERN KENYA (*VERSION 3.0, 230614*)**

Reference is made to your letter dated June 23rd 2014. The ERC Secretariat acknowledges receipt of the revised document on 25 June 2014.

This is to inform you that the Ethics Review Committee (ERC) reviewed the documents submitted, and is satisfied that the issues raised at the 227th meeting, have been adequately addressed.

This study is granted approval implementation effective this **July 1, 2014.** Please note that authorization to conduct this study will automatically expire on **June 30, 2015**. If you plan to continue with data collection or analysis beyond this date please submit an application for continuing approval to the ERC secretariat by **May 19, 2015.**

You are required to submit any amendments to this protocol and other information pertinent to human participation in this study to the SSC and ERC for review prior to initiation.

You may embark on the study.

Yours faithfully,

**DR. ELIZABETH BUKUSI,**
**ACTING SECRETARY,**
**KEMRI/ETHICS REVIEW COMMITTEE**

In Search of Better Health

Con/1002

15 JUN 2015

KISUMU

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**KEMRI/RES/7/3/1** **11th June, 2015**

**TO:** **ERIC OCHOMO,**
**PRINCIPAL INVESTIGATOR**

**THROUGH:** **DR. STEPHEN MUNGA,**
**DIRECTOR, CGHR,**
**KISUMU**

FORWARDED
DIRECTOR
CENTRE FOR GLOBAL HEALTH RESEARCH

Dear Sir,

**RE:** **SSC PROTOCOL NO. 2776 (*REQUEST FOR ANNUAL RENEWAL*): MALARIA VECTOR SURVEILLANCE IN THE CONTEXT OF ENHANCED MALARIA CONTROL IN WESTERN KENYA**

Thank you for the continuing review report for the period **1st July 2014 to 13th May 2015.**

This is to inform that during the 240th A meeting of the KEMRI Scientific and Ethics Review Committee held on 9th June 2015, the Committee **conducted the annual review and approved** the above referenced application for another year.

This approval is valid from **June 9, 2015** through to **June 8, 2016**. Please note that authorization to conduct this study will automatically expire on **June 8, 2016**. If you plan to continue with data collection or analysis beyond this date please submit an application for continuing approval to the **SERU** by **27th April 2016.**

You are required to submit any amendments to this protocol and other information pertinent to human participation in this study to SERU for review prior to initiation.

You may continue with the study.

Yours faithfully,

EAB

**PROF. ELIZABETH BUKUSI,**
**ACTING HEAD,**
**KEMRI/SCIENTIFIC AND ETHICS REVIEW UNIT**

**Appendix IV: Mosquito Direct Skin Feeding informed Consent Form**

Participant Information Sheet

**Title: Determination of influence of blood meal on susceptibility to pyrethroids in *Anopheles gambiae* from Western Kenya.**

**List of investigators**

Maxwell Machani (Bsc)[1,2], David Sang (PhD)[2], Erick Ochomo (PhD)[1], Yaw Afrane (PhD)[1],

Institutions

1. **Error! Reference source not found.**Maseno University - School of public health.

**Purpose of research study**

The main aim of this research is to dertemine the effects of blood meal on the susceptibility to deltamethrin in *anopheles gambiae* mosquitoes one of the main malaria vector in Western Kenya.

**What we will do**

One week from today, we will ask you to spread out your arm and then place it in a cage containing about **100** mosquitoes for about 20 minutes. The mosquitoes will bite you, which will be a little uncomfortable. It will feel similar to you getting bitten by mosquitoes in your house or outdoors. However there is no risk that you will develop an infection or malaria because the mosquitoes are reared in our laboratories and are free of disease. Further, the mosquitoes only take very small volumes of blood. In total the mosquitoes will take less than a teaspoon of blood. We will only need to do this twice a week.

**Potential benefits**

There will be no potential benefit to yourself, but your contribution will help advance the science of malaria. The information obtained will be key to understanding factors contributing the resistance that may lead to inefficiency of the insecticides available and also in developing novel strategies to restore the efficacy of insecticides to minimize its impact on disease control interventions

**Potential risks**

There may be itchiness at the site of mosquito feeding but this will disappear in a few hours.

**Privacy and Confidentiality:** We will protect your privacy. Your name will not be in any reports or journals.

**Reimbursements:** There are no costs to be in this activity and therefore no form of reimbursements.

**Your rights to participate, say no, or withdraw**
You are free to choose to be part of this study. You have the right to refuse. If you do not want to go on with this part of the study, you can stop at any time.

**Contact information for questions or concerns**
If you have any questions about this study, you can contact Dr Yaw Afrane at KEMRI, P.O. Box 1578-40100, Kisumu, . If you have any questions about your rights as a study participant, or if you want to talk about the study with someone who is not directly involved with this study, please contact The Secretary, **KEMRI Scientific and Ethics Review Unit (SERU)**, PO Box 54840-00200, Nairobi; Telephone numbers:. Email address: seru@kemri.org. You may also contact the Secretary or Chairman of the same committee if you feel you have been harmed by this study. If you do not have access to a telephone, or you do not know how to read and write, this will not stop you from participating in this study.

**Consent**

**Name of Volunteer ---------- Age---------- Sex----------**

I have been informed about a study entitled **"Determination of influence of blood meal on susceptibility to pyrethroids in *Anopheles gambiae* from Western Kenya"**. I was requested to participate in direct feeding of mosquitoes using my arm. I understand that I am free to choose to be in this study and that saying "NO" will have no effects. It is therefore, with full understanding of the situation that I gave my informed consent to participate in the study.

Name (Volunteer) ----------Signature ----------Date ---------
Name (investigator) ----------Signature ----------Date ----------
Name (Witness) ----------Signature ----------Date ----------

***Thank you for your participation!***